Stress Hormones and their Regulation in a Captive Dolphin Population

Cory D Champagne & Dorian S. Houser
National Marine Mammal Foundation
2240 Shelter Island Dr, Suite 200
San Diego, CA 92106
phone: (707) 321-6113 fax: (877) 773-3153 email: cory.champagne@nmmf.org
phone: (877) 360-5527 x112 fax: (877) 773-3153 email: dorian.houser@nmmf.org

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LONG-TERM GOALS

The Navy requires an understanding of how markers of stress relate to marine mammal health. This information will inform Navy environmental stewardship efforts and will guide decision making regarding Navy actions with the potential to affect the health of wild marine mammal populations. The research program reported here aids our understanding of how the stress response operates in marine mammals by evaluating markers of stress in a captive dolphin population. It determines baseline levels of putative stress hormones and evaluates the functional consequences of increased stress in the bottlenose dolphin (*Tursiops truncatus*) through the assessment of non-traditional biochemical markers.

OBJECTIVES

The specific research objectives of this effort are to (1) establish protocols for improved sensitivity of low-level corticosteroids (cortisol and aldosterone) frequenctly observed in cetaceans; (2) determine the regulatory role of corticosteroid binding globulin (CBG) in corticosteroid action; (3) assess the role of reverse triiodothyronine (rT_3) in the counter-regulation of thyroid hormone action; and (4) determine the impact of hormone variation associated with the stress-response on the function of metabolic pathways using metabolomic analyses.

APPROACH

Key Individuals and Collaborations
This research project leverages another ONR-funded effort investigating stress markers in bottlenose dolphins at the U.S. Navy Marine Mammal Program (MMP) and is composed of two broad components: 1) assessing baseline variability in stress hormones and 2) evaluating physiological and metabolic alterations that occur during stress. This grant (#N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*; PI: Dorian Houser, PhD; National Marine Mammal Foundation), is hereafter referred to as the *Parent Project*. Hormone assays are being conducted in collaboration with Dr. Daniel Crocker (crocker@sonoma.edu) at Sonoma State University; Department of Biology, Rohnert Park, CA; 94928. Metabolomic sample processing is

being conducted by *Metabolon, Inc*. and in consultation with the Science Development Director, Jeff Buckthal, PhD (JBuckthal@metabolon.com).

Study Approach
This study capitalizes on three experimental components of the Parent Project. (1) Normal variation in stress and metabolic hormones is being evaluated by collecting samples from dolphins throughout the year (*temporal and demographic variation*). Thirty dolphins were sampled to assess temporal and demographic variation in hormone levels. To evaluate the sensitivity of hormone axes, hormone stimulation experiments were conducted on the (2) HPA axis, and on the (3) HPT axis (*HPA* and *HPT stimulation* studies, respectively). During these stimulation experiments, an animal's hormonal and physiological response to a simulated stressor can be evaluated. The HPA axis was first stimulated in bottlenose dolphins via an ACTH injection, and subsequently with an out-of-water stress protocol. The observed response to the stress protocol was similar to that of ACTH administrations (see Parent Project for further details). The HPT axis was activated using thyroid-stimulating hormone (TSH) in a separate set of experiments. The project described here extends the suite of biomarkers assayed in the Parent Project and attempts to improve on processing methods in order to improve quantification of certain stress biomarkers. Four project tasks are being conducted.

Task 1—Improved quantification of circulating corticosteroids
Bottlenose dolphins have low circulating levels of corticosteroids (cortisol and aldosterone). Accurate quantification requires highly sensitive assays to detect variation at or near the typical detection limit of most commercially available immunoassay kits. By modifying existing protocols, this project is evaluating assay techniques to determine the most efficient, reliable, and cost-effective means of measuring circulating corticosteroids in bottlenose dolphin.

Task 2—Assessment of corticosteroid binding globulin
Most corticosteroids in circulation are bound with a carrier protein, primarily corticosteroid binding globulin (CBG). Only unbound hormones, however, are thought to interact with receptors and elicit a response at target tissues. Consequently, variation in carrier proteins like CBG can mediate the metabolic influence of hormones. CBG may in fact be an accurate marker of long-term stress as it does not seem to vary with acute stress in some species (Chow et al, 2010). This project will therefore assess temporal variation in CBG concentration in the bottlenose dolphin.

Task 3—The influence of the HPT axis on rT_3
Variability in, and sensitivity of, the HPT axis is being investigated in the Parent Project. Under stress conditions, rT_3 production can be increased, leading to reductions in energy use by blocking T_3 receptors (Weissman, 1990). This resultant reduction in energy use may be an important energy conserving mechanism necessary to endure stressful periods. We are therefore quantifying rT_3 concentrations for normal variation and during stimulation of the HPA and HPT axes.

Task 4—Functional analysis using metabolomics
The principal role of hormones is to influence metabolic pathways. There are numerous metabolic pathways and thousands of resultant compounds that are likely influenced by hormones associated with the stress response. Many of these compounds can be simultaneously identfied using a broad-based metabolomics technique and the identified compounds can be associated with up- and down-regulation of associated metabolic pathways (Goodacre, et al, 2004) thereby establishing some of the metabolic consequences of stress. We are therefore conducting metabolomic analyses of the Parent

Project HPA and HPT axes stimulations to evaluate the functional consequences of increased stress in the bottlenose dolphin.

WORK COMPLETED

Task 1—By modifying the protocols to commercially available RIA kits (from Seimens, Inc; Washington D.C.) we developed reliable means to assess low concentrations of corticosteroids in bottlenose dolphins and implemented these assays for a subset of samples from the Parent Project (see Figure 1). Unfortunately, during the course of this investigation, the assay kits were discountinued by the manufacturer necessitating a new search, test, and validation of suitable assay kits which has delayed the completion of this task. We have found another supplier (MP Biomedicals; Santa Ana, CA) of appropriate assay products for each of cortisol and aldosterone measurements and these assays are currently being conducted. We anticipate completing this task in the fall of 2015.

Task 2—At the last ONR program revew we consulted with Dr. R. Boonstra regarding CBG assays. His lab has developed reliable and accurate techniques for measuring circulating CBG concentrations from various species and generously agreed to assist with this work. We are therefore collaborating with Dr. Boonstra's laboratory to conduct these measurements rather than developing new methods. CBG assays will begin in the fall of 2015.

Task 3—All rT3 assays have been completed; rT3 concentration was evaluated for seasonal variability (year 1 of the Parent Project, 470 samples from 21 dolphins) and in response to HPT axis stimulation (55 samples from five dolphins).

Task 4—HPA and HPT axes stimulations have been completed in the Parent Project. Both the hormone and metabolite responses to the HPA axis stimulation have been characterized. A total of 454 compounds were identified; these data are currently being analyzed and preliminary results were accepted for presentation at the 21st Biennial Conference on the Biology of Marine Mammals (Dec 2015). HPT axis stimulations were completed in June of 2015 and the hormone assays are currently being conducted. Samples were sent for metabolomics processing and results are anticipated in November, 2015.

RESULTS

Task 1—We established a reliable method of measuring corticosteroids from dolphin serum at low circulating concentrations. We modified a protocol from a simple, affordable, and commercially available RIA kit (Siemens coat-a-count TKCO1) and conducted a validation of the RIA using serially diluted dolphin serum samples and found excellent parallelism with the standard curve. We determined that there were no interferring substances in dolphin samples by comparing steroid-extracted (purified) and non-extracted serum samples and found excellent agreement between the two measurements (± 9%) across a four-fold dilution. There was no detectable cortisol present in steroid-stripped serum, indicating there is no cross-reactivity with the assay antibody and polar matrix compounds. This cortisol kit, however, has since been discontinued by the manufacturer. Other manufacturers are increasing product lines to fill this need and assay kit options are currently being explored, primarily from MP Biomedicals (Santa Ana, CA).

Similar to the cortisol determinations, we also established a method of measuring low concentrations of aldosterone from dolphin serum (see Figure 1C). The first procedure used a coated-tube assay kit

that was discontinued by the manufacturer (Seimens, Inc) during the course of the study. We selected a replacement kit from MP Biomedicals (catalog # 07-108202) that first uses an extraction step and subsequent dual-antibody RIA and we have successfully used this kit to measure very low aldosterone concentrations in dolphin serum samples. The extraction can be conducted with varying volumes of serum (~1 mL) and extracts aldosterone into an organic phase of 3 parts ethyl acetate to 2 parts hexane. The extracted hormone solution is dried and reconstituted in a constant volume of assay matrix. This flexible procedure is easily modified and should be readily applicable to a range of aldosterone concentrations found among marine mammals and appropriate for use across laboratories. With the initial peer-reviewed publication from this project we will promulgate the cortisol and aldosterone assay protocols in *supplementary material* to promote consistency in hormone assays within the marine mammal field.

Corticosteroid concentrations were low in this managed dolphin population (mean cortisol concentrations were 18.1 (sd 11.0) and 16.8 (sd 10.2) nM in years 1 and 2, respectively). Some of these values, however, may be artificially low due to treatment with megesterol acetate (MegAce) in some study subjects (Champagne et al., 2013 and see report for Grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population* for details). Although consistently low compared to other studies (e.g. Ortiz & Worthy, 2001), cortisol concentrations varied daily, seasonally, and with sex (see Figure 1).

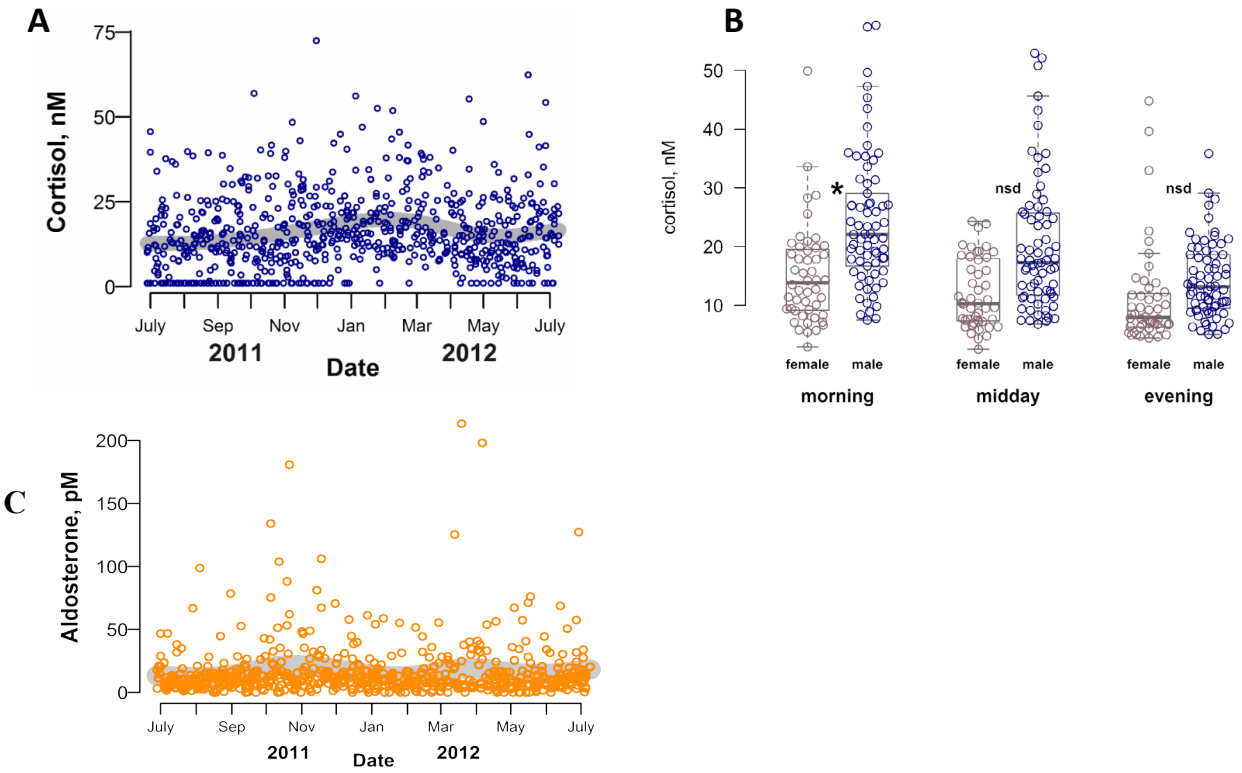

Figure 1. Circulating corticosteroid concentrations in U.S. Navy MMP dolphins. (A) Circulating cortisol concentrations were consistently low among 30 captive dolphins; data from 731 samples collected between July 2011 – 2012. (B) Cortisol concentrations varied daily and by sex – concentrations were highest in the morning in both sexes and varied by sex in the morning (asterisk* indicates significant difference while "nsd" indicates no significant difference between sexes within sample time). (C) Circulating aldosterone concentrations were low but variable among the study population (mean 16.2, sd 21.5 pM; data from 624 samples from 26 dolphins).

Task 3—Average rT3 concentration was 6.75 nM (sd 1.91, min 2.19, max 13.69) throughout year 1 of the parent project (see Figure 2). These values are somewhat higher than previously reported in other odontocetes (e.g. St. Aubin *et al* 2013) but these moderate differences are not surprising given the differences in study groups, methods of sample collection, and laboratory assessment among published studies. Our findings provide further support that rT3 concentration is generally quite high in marine mammals (including odontocetes and pinnipeds; Atkinson *et al* 2015; Champagne *et al* 2015) when compared with terrestrial mammals (e.g. the reference range in humans is 0.1 – 0.4 nM; Moore & Eastman, 1990). These data will be combined with other measurements from the parent project to evaluate relationships between hormones of the HPT axis and interactions with the HPA axis.

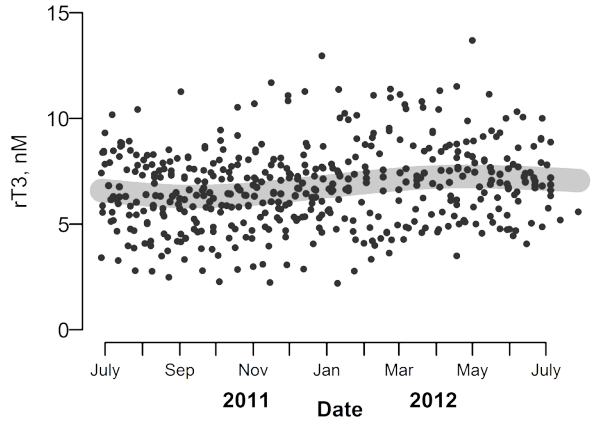

Figure 2. Relative to terrestrial mammals, circulating rT3 concentrations were consistently high among 21 captive dolphins managed by the U.S. Navy MMP (mean 6.75, sd 1.91 nM; data from 470 samples collected between July 2011 and July 2012).

Task 4—Increases in circulating ACTH and cortisol concentrations are consistent with an acute stress response and associated with increased aldosterone concentrations (see report for Grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*). Characterization of changes in the circulating metabolome revealed increases in other glucocorticoids—cortisone and corticosterone. The stress response altered circulating concentrations of several metabolites, including markers of carbohydrate metabolism (glucose, lactate, and pyruvate) typical of the mammalian stress response. Markers of lipid mobilization (e.g. glycerol, medium, long, and polyunsaturated fatty-acids) and β–oxidation (acylcarnitines) also increased during stress suggesting an important role of lipid use during the stress response in this taxa (see Figures 3 & 4).

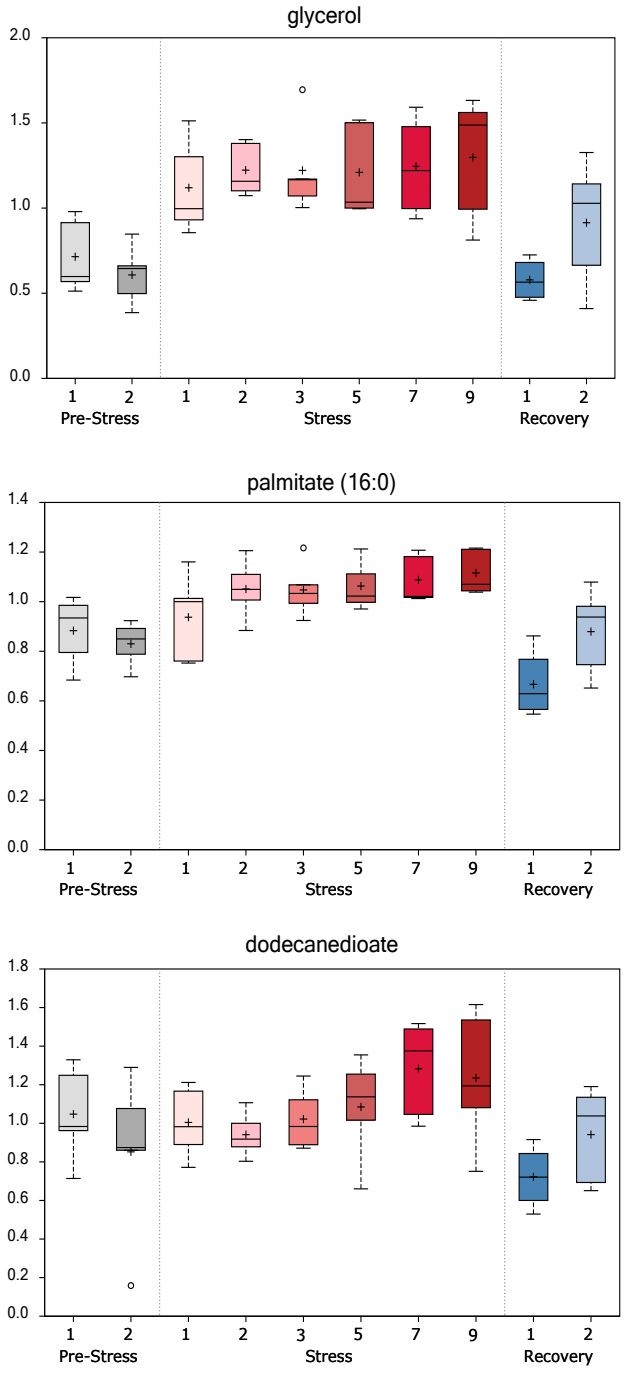

Figure 3. Markers of lipolysis increased during acute stress, including glycerol, medium, long, and polyunsaturated fatty acids (e.g. palmitate and dodecanedioate).

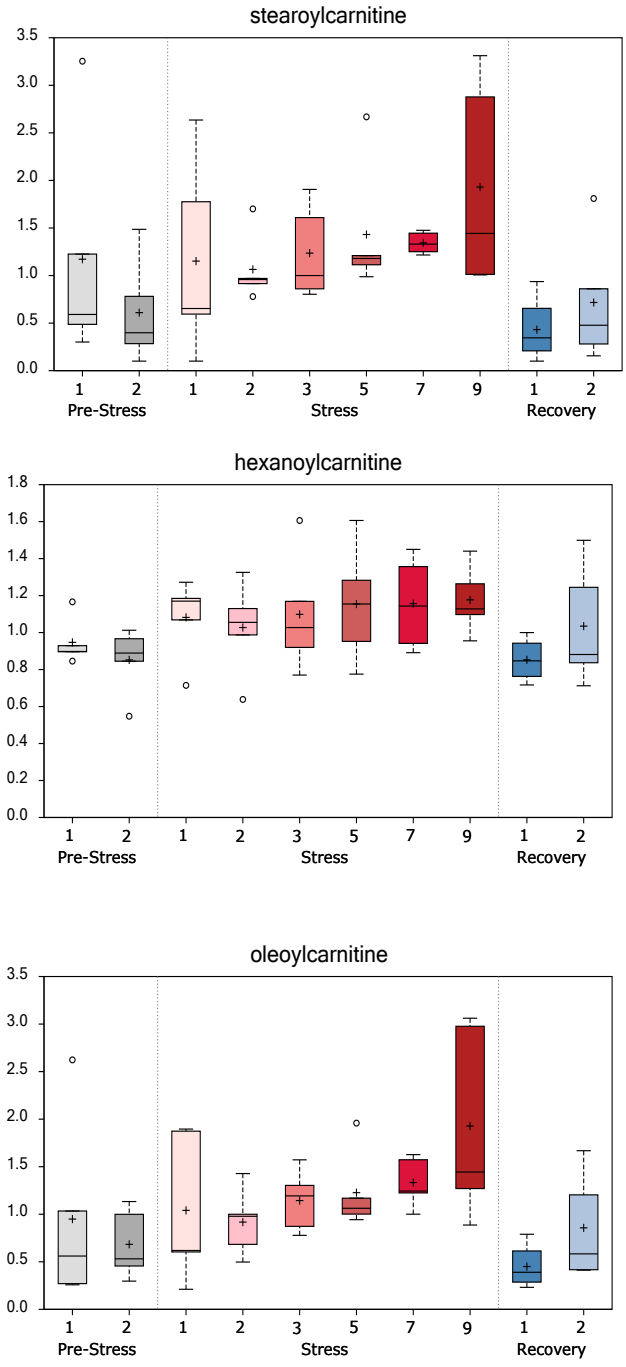

Figure 4. Several acylcarnitines (e.g. stearoylcarnitine, hexanoylcarnitine, and oleoylcarnitine) were elevated during acute stress, suggesting increased fatty-acid transport into mitochondria for β-oxidation.

IMPACT/APPLICATIONS

Marine mammals negatively influenced by acoustic disturbances or other U.S. Navy activities potentially experience a "stress response." The stress response can be detected by changes in stress markers, including select hormone concentrations, alterations in metabolic pathways, and potentially certain metabolite levels. The stress response can influence survival and reproduction and, therefore, may have population-level effects (Wikelski & Cooke, 2006). The additional characterization of hormones, hormone regulators, and metabolites during baseline and simulated stress conditions, as described in the current proposal, provides a mechanism by which to better detect the presence and magnitude of the physiological responses of marine mammals exposed to anthropogenic stressors. In accordance with National Research Council recommendations (2005), the work described in this proposal, in concert with the Parent Project, will establish baseline and activated levels for putative stress markers in marine mammals.

RELATED PROJECTS

Grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*; PI: Dorian Houser, PhD; National Marine Mammal Foundation. This project is the parent project from which samples have been collected.

Grant #N000141512214, *Quantifying stress in Marine Mammals: measuring biologically active cortisol in cetaceans and pinnipeds*; PI: Rudy Boonstra, PhD; University of Toronto. This project looks to characterize the equilibrium dissociation constant for CBG as well as the CBG binding capacity of plasma for a number of marine mammal species.

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PUBLICATIONS

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